



# The effect of salinity on the survival, growth, sporulation and infection of *Phytophthora ramorum*



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## ARTICLE INFO

### Article history:

Received 5 January 2016

Received in revised form

28 July 2016

Accepted 2 August 2016

Corresponding Editor: Ayco J. M. Tack

### Keywords:

Brackish water

Epidemiology

*Phytophthora ramorum*

Ramorum blight

Salinity

## ABSTRACT

*Phytophthora ramorum* has been found in waterways outside infested nurseries, but little is known about its behavior in water. This study examined the effect of salinity on survival, growth, sporulation, and infection. *P. ramorum* survival and growth was negatively correlated with salt concentration (range of 0–45 g l<sup>-1</sup>), but showed a level of tolerance even at 45 g l<sup>-1</sup>. No sporangia were observed in cultures with higher than 20 g l<sup>-1</sup> of salt and zoospores were not released from sporangia above 14 g l<sup>-1</sup>. Water sources with different salinity were used to understand the environment where *P. ramorum* can survive and infect host material. Water from natural bodies and water amended with different salt concentrations were added to *P. ramorum*-infested sand and baited with rhododendron leaf disks. Infection decreased with increasing salt concentration and increased with higher initial concentration of *P. ramorum*. This research helps to better understand the effects of water quality on survival and infectivity of *P. ramorum*, expanding the potential survey range.

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## 1. Introduction

*Phytophthora* species are grouped in the Oomycota and are often referred to as water molds (Blackwell, 1944). As this name reflects, oomycetes are often associated with moisture and, in general, free water highly favors *Phytophthora* spp. infection of plants. Although the vast majority of known *Phytophthora* spp. are plant pathogens, some recently discovered and identified species have been proposed to have a saprotrophic lifestyle as a rapid colonizer of leaf litter or the very least opportunistic pathogens (Brasier et al., 2003; Hansen et al., 2012). A majority of those species have been found in riparian ecosystems. However, very little is known about their survival and proliferation in this environment.

*Phytophthora ramorum* is a pathogen that persists and spreads on a wide range of ornamentals. It has been found in irrigation ponds and ditches within nurseries (Jeffers et al., 2010), waterways

outside of nurseries (Chastagner et al., 2010), and in streams in forested habitats where *P. ramorum* is known in the natural environment (Sutton et al., 2009). Despite this information, very little is known about the effect of the water characteristics on survival of *P. ramorum* and *Phytophthora* spp. in general. Chandler et al. (2006) evaluated the effect of water temperature and bacteria on sporulation of *Phytophthora alni* in river water, while Werres et al. (2007) concluded that water temperature was a major influencing factor in production of *P. ramorum* sporangia. In addition, Kong et al. (2012b) found that *P. ramorum* was tolerant to an aquatic environment with a pH range of 5–11. In a related study (Kong et al., 2012a), a higher total salt concentration, as measured by electrical conductivity, stimulated growth and sporulation of *P. ramorum*. One factor in water quality that has not been investigated fully for its effect on *Phytophthora* spp. is salinity, which is related but not the same as electrical conductivity.

In 1990, nine marine *Phytophthora* spp. were reclassified in the genus *Halophytophthora* based upon their differences in morphological and cultural characteristics (Ho and Jong, 1990). This was later confirmed in a study comparing the ITS sequences of genomic rDNA (Cooke et al., 2000). *Halophytophthora* spp., which are closely related to *Phytophthora* spp., have been found primarily in marine ecosystems with only a recent discovery of a freshwater species (Yang and Hong, 2014). *Halophytophthora* spp. have a wide

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tolerance to salinity ranging from fresh to brackish water (Leaño et al., 2000; Nakagiri, 2000). Recently, two *Phytophthora* spp., *Phytophthora gemini* sp. nov. and *Phytophthora inundata*, were isolated from seagrass (*Zostera marina*) in a brackish-saline environment (Man in 't Veld et al., 2011). However, no in-depth studies related directly to the salt tolerance of these two species or any *Phytophthora* spp. could be found. Indirect studies related to disease and soil salinity have demonstrated an effect (Blaker and MacDonald, 1986; Swiecki and MacDonald, 1991), which is often related to salt stress on the plant itself (Snapp et al., 1991). Several studies have demonstrated that although an increase in soil salinity stimulates sporangia formation of different *Phytophthora* spp., zoospore release and activity is reduced (Blaker and MacDonald, 1985; Swiecki and MacDonald, 1991). Some fungi have been found to have a high salt tolerance (Tresner and Hayes, 1971). The level of salt tolerance is important to know for *P. ramorum* to help understand how this pathogen may potentially spread. Understanding the effect of salinity on survival, growth, proliferation, and infection of *P. ramorum* would lead to more knowledge of the potential habitat range and more efficient surveys. Some preliminary results have been reported previously (Preuett et al., 2011).

## 2. Methods

### 2.1. *Phytophthora ramorum* isolates

Six different *P. ramorum* isolates: WSDA-1772 and 5-C (A2 mating type and clonal lineage NA1), PRN-1 and BBA/15 (mating type A1 and clonal lineage EU1), and 09-053 and 05-166 (unknown mating type and clonal lineage NA2) were cultured on 20% clarified V8 agar. The isolates were maintained on autoclaved rye seed for long term storage (Peters et al., 1998).

Mycelium-free chlamydospores of the six *P. ramorum* isolates were produced by a modified method described by Mitchell and Kannwischer-Mitchell (1992) and stored in autoclaved sand at 4 °C as described by Widmer et al. (1998). Five agar plugs (5-mm diameter), containing actively growing mycelium of each of the *P. ramorum* isolates, were placed separately in 20 ml of 20% sterile, clarified V8 broth in sterile Petri plates (100 mm diameter) and stored at 20 °C in the dark. After 4 weeks, the mycelia and chlamydospores were transferred to a blender cup containing 50 ml of sterile water and blended for 20 s. The suspension was mixed with autoclaved masonry sand and stored at 20 °C for at least 4 weeks until the mycelium was no longer viable. The concentration of the chlamydospores in the sand was measured by diluting 5 cm<sup>3</sup> of the infested sand in 95 ml of 0.2% water agar and plating 1 ml on *Phytophthora*-selective medium (PARPH+V8; Ferguson and Jeffers, 1999). The *P. ramorum* colonies were counted after 3 d and the chlamydospore concentration cm<sup>-3</sup> of sand was calculated based on the average over 10 plates. The sand inoculum was stored at 4 °C for long term storage until it was ready to be used.

*P. ramorum* sporangia and zoospores were produced by the method described by Widmer (2009). Three agar plugs (5 mm diameter), containing actively growing mycelium of each of the *P. ramorum* isolates, were placed separately in 16 ml of 10% sterile, clarified V8 broth in sterile Petri plates (100 mm diameter) and stored at 20 °C under continuous light (3000 lux). After 5 d, a mycelium-free suspension of sporangia were collected by vigorously shaking the cultures in a sterile conical tube and filtering through two layers of sterile cheesecloth. Zoospores were produced by exposing the sporangia suspension to 4 °C for 1 h and then letting it sit for 45 min at room temperature (20–25 °C). The zoospore suspension was filtered through a 30 µm screen and encysted by vortexing.

### 2.2. Collection and preparation of water samples

Salt solutions of 6, 14, 20, 35, and 45 g l<sup>-1</sup> were prepared in the laboratory by dissolving natural white sea salt (La Baleine, Aigues-Mortes, France) in deionized water in a volumetric flask. Salt concentrations of 6, 14, 20, 35, and 45 g l<sup>-1</sup> were chosen based upon concentration ranges of saline water found in nature and defined by the United Nations Food and Agriculture Organization (FAO, 1992). The saline water ranges for the salt solutions represent moderately saline, highly saline, highly saline, very highly saline, and brine, respectively.

Water samples from Lake Pontchartrain (Slidell, LA; 30° 13' 07.01" N; 89° 49' 23.00" W), Atlantic Ocean (near Kill Devil Hills, NC; 35° 59' 08.12" N; 75° 38' 19.07" W), Chesapeake Bay (Norfolk, VA; 36° 56' 39.86" N; 76° 13' 59.87" W), and York River (Yorktown, VA; 37° 14' 01.79" N; 76° 30' 12.89" W) were collected by dipping a clean container directly in the water source at the exact points and transferring to a clean glass container that was placed in a cooler until it could be refrigerated. All samples were filtered prior to use through a 5.0 µm, hydrophilic membrane filter using Millipore 47 mm glass vacuum filtering system (Millipore, Inc., Billerica, MA). The water solutions were placed in autoclaved bottles after filtering and stored at 4 °C until they could be used. The pH and conductivity were measured and recorded at room temperature.

### 2.3. Effect of salinity on growth and sporulation of *Phytophthora ramorum*

The effect of salinity on the growth of *P. ramorum* was conducted in two different studies. The first study examined mycelium growth as indicated by the dry weight after growing in liquid broth. A sterile 10% clarified V8 broth with final salt concentration of 0, 6, 14, 20, 35, or 45 g l<sup>-1</sup> were prepared by mixing 3 ml of an autoclaved 20% clarified V8 broth with 3 ml of an autoclaved salt solution of 0, 12, 28, 40, 70 or 90 g l<sup>-1</sup>, respectively, in a 60 mm diameter Petri plate. An agar plug (3 mm diameter) containing actively growing hyphae from the advancing edge of a *P. ramorum* colony on V8 agar for each of the six *P. ramorum* isolates tested was transferred to each plate. Controls were included for each salt concentration by adding only a V8 agar plug. There were six plates of each isolate for each salt concentration. Three of the plates for each isolate were placed in a 20 °C incubator under continuous light (3000 lux) to induce sporangia production (Widmer, 2010), while the remaining three plates were wrapped and placed in a 20 °C incubator in the dark for mycelium growth. After 1 week, the plates were removed from the incubator and the contents of one plate was poured onto a pre-weighed Whatman™ 1 filter paper (90 mm diameter; GE Healthcare UK Limited, Buckinghamshire, UK) sitting under slight vacuum on a Buchner filter. The filter paper and mycelium were dried under a laminar flow hood for 4 h. The filter paper was weighed and the pre-weight subtracted to obtain the preliminary weight of mycelium. The final weight of the mycelium was obtained by subtracting the average preliminary weight of the corresponding controls for that salt concentration from the preliminary weight of the mycelium. The experiment was conducted twice.

The second study examined the effect of agar plates amended with various concentrations of salt on colony growth. Agar plates were prepared by mixing equal volumes of 40% clarified V8 broth and salt solutions of 12, 28, 40, 70, or 90 g l<sup>-1</sup> (for final salt concentrations of 6, 14, 20, 35, and 45 g l<sup>-1</sup>, respectively) with 18 g l<sup>-1</sup> agar and autoclaving for 20 min. After cooling to 50 °C, the agar slurry was poured into 60 mm diameter Petri plates and allowed to solidify. An agar plug (3 mm diameter), taken from the edge of an actively growing culture of the tested *P. ramorum* isolate, was placed in the center of an agar plate at each salt concentration,

including a control of only 20% V8. Three plates were prepared for each isolate at each salt concentration. The plates were wrapped to avoid drying and placed in a 20 °C incubator in the dark. Colony growth was measured after 3 and 7 d by marking the leading edge of the colony at four points perpendicular to each other intersecting through the middle of the plug. The experiment was conducted twice.

To measure the production of sporangia, the plates under continuous light, prepared above, were removed after 5 d and the total contents transferred to a 15 ml conical tube. The tube was vortexed for approximately 15 s to release the sporangia into suspension. Immediately after vortexing, the contents were filtered through cheesecloth into a clean 15 ml conical tube where the volume was recorded. The concentration of sporangia in suspension was calculated by averaging the counts from three 20 µl samples. For uniformity, the number of sporangia per 10 ml suspension was calculated for each repetition. The experiment was conducted three times.

To determine the effect of salinity on zoospore release, sporangia for each of the isolates were produced as described above in twelve Petri plates (60 mm diameter) containing 6 ml of 10% V8 broth. After 5 d, the V8 broth was removed and replaced with 6 ml of sterile water plus salt at concentrations of 0, 6, 14, 20, 35, and 45 g l<sup>-1</sup> so that there were two replications per isolate for each salt concentration. The plates were placed in a 4 °C incubator for 30 min and then placed at room temperature. After 1 h, the cultures were observed for zoospore release. Afterwards, the plates were returned to the 20 °C incubator under continuous light for 24 h. The cultures were observed again for zoospore activity and germination of the sporangia. The experiment was conducted twice.

#### 2.4. Effect of salinity on propagule survival of *Phytophthora ramorum*

Chlamydospores, sporangia and encysted zoospores were produced as described above. Chlamydospores were separated from the larger particles of sand by mixing an equal portion of sand inoculum with sterile 0.1 mM MES buffer, pH 6.2, shaking vigorously for 10 s, allowing the larger sand particles to settle for 2 s, and filtering the solution through a 100 µm mesh screen. The suspensions of each of the spore types were centrifuged for 10 min at 2053 g. The supernatant was removed and the resulting pellet was resuspended in 6 ml of sterile 0.1 mM MES buffer, pH 6.2. One milliliter of this suspension was transferred into 6 sterile 1.8 ml microfuge tubes and centrifuged for 2 min at 21,130 g. After removing the supernatant, the pellets were resuspended in 1 ml sterile water or salt solutions of 6, 14, 20, 35, or 45 g l<sup>-1</sup>, prepared as described above. The resulting chlamydospore suspension was placed at 4 °C in the dark to limit germination in the solution (Tooley et al., 2014) and the sporangia and encysted zoospore suspensions had to be placed at 20 °C in the dark, to limit zoospore formation and release of the sporangia so that only direct germination would be observed.

After 24 or 48 h at 20 °C and 7 d at 4 °C, the tubes containing the sporangia or encysted zoospore and chlamydospore suspensions, respectively, were removed and centrifuged for 2 min at 21,130 g. The supernatant was removed and the pellet was resuspended in 1 ml of sterile 0.1 mM MES buffer, pH 6.2. After mixing well, the suspensions were poured onto the surface of solidified PARPH+V8 medium in a 100 mm diameter Petri plate. After the liquid was allowed to evaporate by removing the lid in a Class II, Type A laminar flow biosafety cabinet, the plates were placed in a 20 °C incubator. After 24 h, the percent germination was determined by observing 100 randomly chosen spores on each plate and counting those that germinated. A spore was considered

germinated if an emerging germ tube was at least the length of the spore diameter. The experiment was conducted a total of three times for each spore type at each salt concentration.

#### 2.5. Effect of salinity levels on leaf baiting

Autoclaved sand (15 cm<sup>3</sup>) pre-infested with 100 or 1000 chlamydospores cm<sup>-3</sup> of *P. ramorum* isolate WSDA-1772, prepared as described above, was added to plastic cups (266 ml). In each cup, 50 ml of the prepared salt solutions (6, 14, 20, 35, and 45 g l<sup>-1</sup>) along with a solution of 0.1 mM MES buffer, pH 6.2, included as a positive control, were added to the cups and allowed to sit until the sand settled. Ten *Rhododendron* 'Cunningham's White' leaf disks (11 mm diameter), cut from leaves collected from greenhouse plants free of visual defects and washed in tap water, were placed abaxial-side down on the water surface in each cup. The cups were covered and placed at 20 °C in the dark. After 1 week, the leaf disks were removed, surfaced sterilized in 70% ethanol for 20 s, rinsed three times in deionized water, and plated on *Phytophthora*-selective medium (PARPH+V8) in Petri plates (90 mm diameter). The Petri plates were placed in an incubator at 20 °C in the dark. The leaf disks were rated positive for infection if *P. ramorum* mycelium was observed emerging from the disks after 5 d. The experiment was conducted three times per treatment.

To determine whether the various salt solutions affect the viability of *P. ramorum* after infection has occurred, two *Rhododendron* 'Cunningham's White' leaf disks (11 mm diameter) artificially-infected with each of the *P. ramorum* isolates were placed in microfuge tubes containing 1 ml of sterile 0, 6, 14, 20, 35, or 45 g l<sup>-1</sup> salt solution with two replications per isolate for each salt concentration. The tubes were placed in a 20 °C incubator in the dark. After 4 and 7 d, one of the disks was removed, rinsed in sterile water and placed on PARPH+V8 medium. Any growth of *P. ramorum* from the leaf disks was rated as positive after 3 d. The experiment was conducted twice.

#### 2.6. Effect of brackish or salt water from natural sources on leaf baiting

Autoclaved sand (15 cm<sup>3</sup>) pre-infested with 1000 chlamydospores cm<sup>-3</sup> of *P. ramorum* isolate WSDA-1772, prepared as described above, was added to plastic cups (266 ml). To each cup, 50 ml of the individual filtered water samples, collected and prepared as described above, were added and allowed to sit until the sand settled. Afterwards, 10 *Rhododendron* 'Cunningham's White' leaf disks (11 mm diameter), cut from leaves collected from greenhouse plants free of visual defects and washed in tap water, were placed abaxial-side down on the water surface in each cup. The cups were covered and placed at 20 °C in the dark. Controls using chlamydospore-infested sand and 0.1 mM MES buffer, pH 6.2 (positive control) or non-infested sand and MES buffer (negative control) were also prepared. After 1 week, the leaf disks were removed, surfaced sterilized in 70% ethanol for 20 s, rinsed three times in deionized water, and plated PARPH+V8 in Petri plates (90 mm diameter). The Petri plates were placed in an incubator at 20 °C in the dark. The leaf disks were rated positive for infection if *P. ramorum* mycelium was observed emerging from the disks after 5 d. The experiment was conducted three times per treatment.

#### 2.7. Statistical analyses

For each of the experiments described above, there was no significant difference ( $P > 0.05$ ) within the replicated, individual treatments; therefore, the data for each treatment could be combined for each of the repeated experiments. The survival of the

different spore types exposed to different salt concentrations was analyzed by regression analysis using SAS for Windows (version 9.2; SAS Inc., Cary, NC). To interpret the overall effect of increasing salt concentrations on *P. ramorum* mycelium weight, colony growth, sporangia production and infection of rhododendron leaf disks, data were analyzed using the regression procedure of Minitab (release 17; Minitab, Inc., University Park, PA). To determine significant differences between the controls (0 g l<sup>-1</sup> salt) and the individual salt concentrations (6, 14, 20, 35, or 45 g l<sup>-1</sup>), the data were analyzed by analysis of variance (ANOVA) and means compared using Tukey's test in Minitab 17.

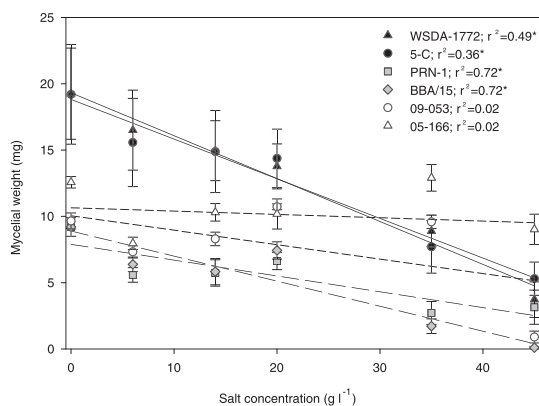
### 3. Results

#### 3.1. Effect of salinity on growth and sporulation of *Phytophthora ramorum*

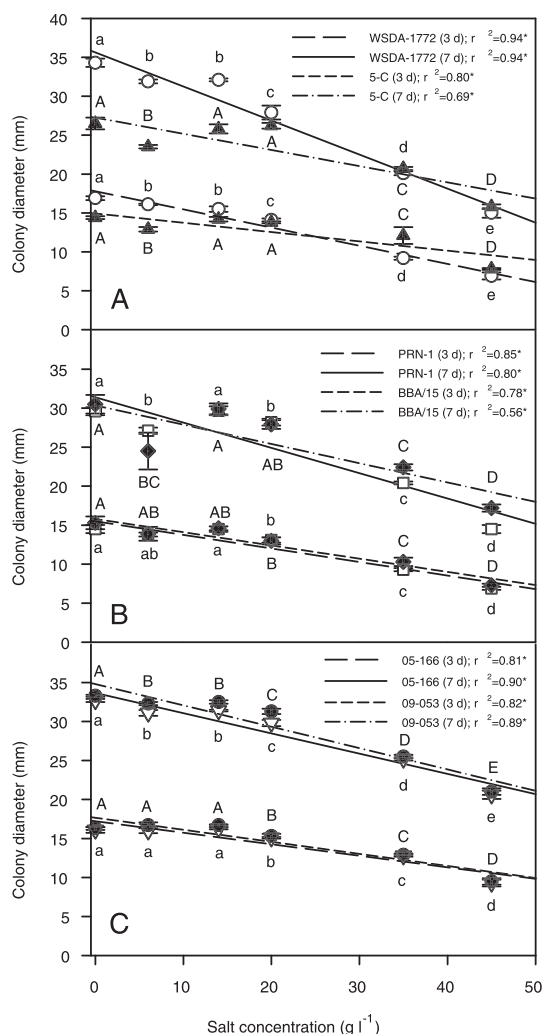
In liquid culture, salt concentration had a significant effect on the mycelium weights of *P. ramorum* NA1 isolates WSDA-1772 ( $P < 0.0001$ ) and 5-C ( $P < 0.0001$ ) and EU1 isolates PRN-1 ( $P < 0.0001$ ) and BBA/15 ( $P = 0.0002$ ), but not for NA2 isolates 09-053 ( $P = 0.368$ ) and 05-166 ( $P = 0.482$ ) (Fig. 1). On agar, salt concentration had a significant effect ( $P < 0.0001$ ) on colony diameter measured after 3 or 7 d for all isolates (Fig. 2). In all isolates after 7 d, the colony diameters grown on agar amended with 14 g l<sup>-1</sup> of salt were not significantly reduced as compared to those grown on 6 g l<sup>-1</sup> and in some isolates to non-amended V8 agar. For three isolates (5-C, PRN-1, and BBA/15) this lack of significance extended out to 20 g l<sup>-1</sup>. Even at the highest salt concentration tested, growth was still evident on agar plates.

When regression lines were compared between the isolates for each lineage, there was a significant difference in colony growth on solid agar between the two NA1 isolates at 3 d ( $P < 0.0001$ ) and 7 d ( $P < 0.0001$ ), but not between the two EU1 isolates ( $P = 0.605$  and  $P = 0.423$ , respectively) or the two NA2 isolates ( $P = 0.378$  and  $P = 0.069$ , respectively). In liquid broth, no differences were observed between the isolates of the NA1 ( $P = 0.784$ ), NA2 ( $P = 0.864$ ), or EU1 ( $P = 0.221$ ) lineages.

In all isolates, sporangia production declined as the salt concentration increased (Fig. 3). In some isolates, sporangia production was not observed at a concentration as low as 14 g l<sup>-1</sup>. Sporangia that formed in broth amended with salt were often elongated with an obvious vacuole compared to those formed in broth alone



**Fig. 1.** Linear regression lines of the dry weight of *Phytophthora ramorum* isolates of NA1 lineage (WSDA-1772 and 5-C), EU1 lineage (PRN-1 and BBA/15), and NA2 lineage (09-053 and 05-166) mycelium after 1 week grown in 20% clarified V8 broth amended with various concentrations of salt. Error bars represent the standard error of the mean (95%). Isolates followed by \* denote significant difference according to regression analysis ( $P \leq 0.05$ ).



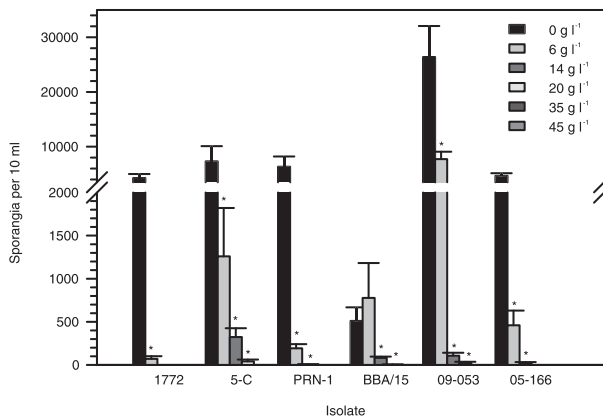
**Fig. 2.** Linear regression lines from colony diameters (mm) of *Phytophthora ramorum* isolates representing (A) NA1 lineage (○-WSDA-1772; ▲-5-C), (B) EU1 lineage (□-PRN-1; ◆-PRG-2), and (C) NA2 lineage (▽-05-166; ●-09-053) after growth for 3 and 7 d on 20% clarified V8 agar amended with various concentrations of salt. Error bars represent the standard error of the mean (95%). Isolates followed by \* denote significant difference of salt effect according to regression analysis ( $P \leq 0.05$ ). Individual points designated by the same letter (lower case for first isolate listed, upper case for second isolate listed) are not significantly different for that isolate at the measured time interval according to Tukey's test ( $P \geq 0.05$ ).

(Fig. 4).

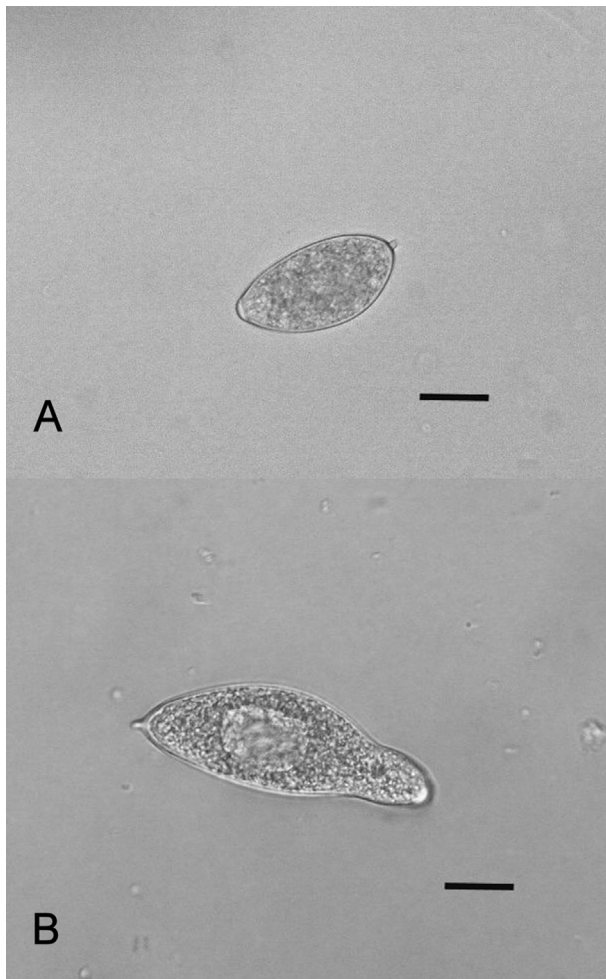
Zoospores were released from sporangia in solutions containing 0–14 g l<sup>-1</sup> salt, but not in salt solutions  $\geq 20$  g l<sup>-1</sup> (data not shown). However, direct germination of sporangia was observed from these sporangia in the higher salt concentration solutions after 24 h. Germination of encysted zoospores and sporangia that did not release zoospores was observed also in the lower salt solutions ( $\leq 14$  g l<sup>-1</sup>).

#### 3.2. Effect of salinity on propagule survival of *Phytophthora ramorum*

Direct germination of chlamydospores, sporangia and encysted zoospores in liquid suspension was observed after exposure to all salt concentrations for the times tested, but the percentage declined as the salt increased (Fig. 5). Analyses of the Order 3 polynomial trendlines verified a significant effect ( $P \leq 0.05$ ) of salt concentration on germination for each spore type.



**Fig. 3.** Sporangia production of *Phytophthora ramorum* isolates of NA1 lineage (1772 and 5-C), EU1 lineage (PRN-1 and BBA/15), and NA2 lineage (09-053 and 05-166) after 5 d grown in 10% clarified V8 broth amended with various concentrations of salt. Error bars represent the standard error of the mean (95%). Bars designated with an \* denote significant difference compared to the control (0 g l<sup>-1</sup>) according to Tukey's test ( $P \leq 0.05$ ). Values of '0' were not analyzed for significance.



**Fig. 4.** *Phytophthora ramorum* sporangium grown in (A) 20% V8 broth or (B) 20% V8 broth plus 14 mg l<sup>-1</sup> salt. Bars = 20 μm.

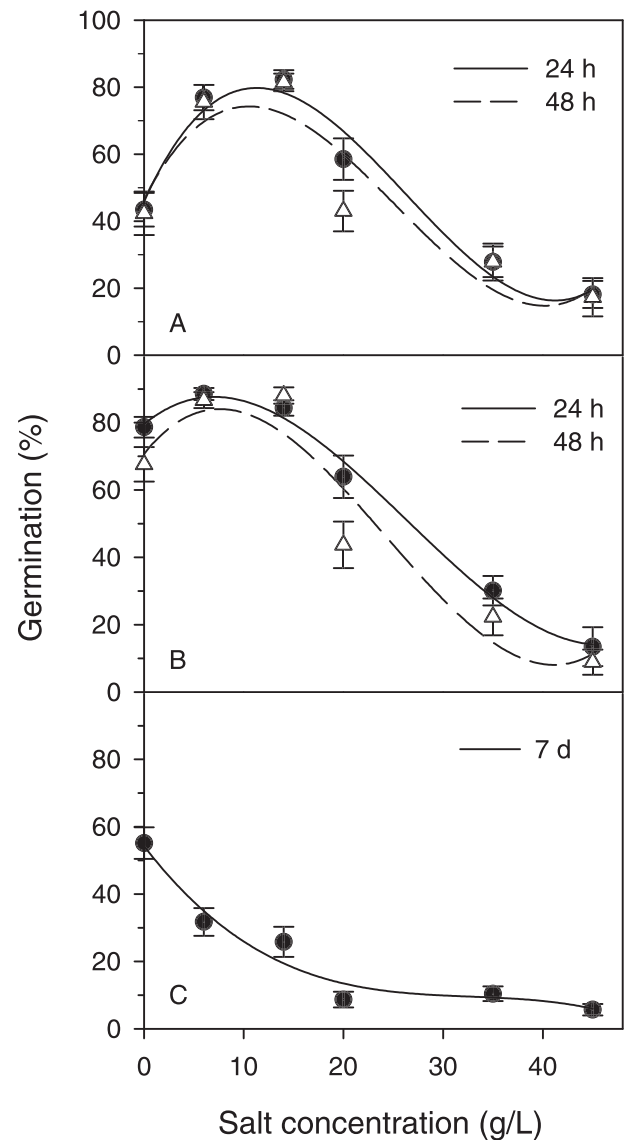
### 3.3. Effect of salinity levels on leaf baiting

Salt concentration had a significant effect on the percentage of *P. ramorum*-positive baits, regardless of the initial concentration of

*P. ramorum* chlamydospores in the sand (Table 1). The initial concentration of *P. ramorum* chlamydospores had a significant effect on the percentage of positive baits at all salt concentrations except 0, 35, and 45 g l<sup>-1</sup>. *P. ramorum* was not recovered from baits at the highest level of 45 g l<sup>-1</sup>, regardless of the initial chlamydospore level. *P. ramorum* was recovered from all samples after exposing artificially infected rhododendron leaf disks to the entire range of salt concentrations tested for 4 and 7 d, regardless of the isolate used (data not shown).

### 3.4. Effect of brackish or salt water from natural sources on leaf baiting

Artificially infested *P. ramorum* sand, mixed with water from different natural sources, baited positive *P. ramorum* samples for all water sources tested (Table 2). However, the percentage of infected bait samples was significantly lower ( $P \leq 0.05$ ) in the very highly saline samples as compared to the controls. Water collected from



**Fig. 5.** Order 3 polynomial trendlines of the percent germination of *Phytophthora ramorum* sporangia (A), encysted zoospores (B), and chlamydospores (C), after exposure to various concentrations of salt solution for designated times. Error bars represent the standard error of the mean (95%).

**Table 1**  
Percentage of *Phytophthora ramorum*-infected *Rhododendron* 'Cunningham's White' leaf disks after exposure for 1 week in water samples amended with different salt concentrations mixed with sand infested with *P. ramorum* chlamydospores at two levels (100 and 1000 cm<sup>-3</sup>).

Salt concentration	Conductivity	pH	Infected leaf disks (%) <sup>a</sup>	
			100	1000
0 (MES buffer)	21.6 µS	6.2	100 ± 0.0	100 ± 0.0
6 g l <sup>-1</sup>	10.3 mS	6.5	61.1 ± 9.9*	100 ± 0.0
14 g l <sup>-1</sup>	26.5 mS	6.7	23.1 ± 3.3*	70.0 ± 6.6*
20 g l <sup>-1</sup>	36.0 mS	7.4	3.3 ± 3.3*	55.6 ± 6.7*
35 g l <sup>-1</sup>	57.2 mS	7.6	0.0 ± 0.0	2.2 ± 2.2*
45 g l <sup>-1</sup>	67.9 mS	9.4	0.0 ± 0.0	0.0 ± 0.0
Regression <sup>b</sup>	—	—	$P < 0.0001$ ; $r^2 = 0.64$	$P < 0.0001$ ; $r^2 = 0.89$

<sup>a</sup> Percentage of rhododendron leaf disks infected with *P. ramorum* after exposure to initial concentrations of 100 or 1000 chlamydospores cm<sup>-3</sup> of sand. Means are followed by the standard error (95%). Values followed by \* denote significant difference compared to the control according to Tukey's test ( $P \leq 0.05$ ). Values of '0.0' were not analyzed for significance.

<sup>b</sup> Significance after regression analysis for each chlamydospore level.

the York River, Chesapeake Bay, and Atlantic Ocean all had conductivities that classified them as very highly saline (FAO, 1992), while water from Lake Pontchartrain was classified as moderately saline.

#### 4. Discussion

Results from this study clearly show that *P. ramorum* can grow and survive in both marine and fresh water habitats, although the attributes tested were all negatively correlated with an increase in salt concentration. For the two NA2 isolates, data between the growth in liquid broth and agar may seem to be contradictory as there was a significant effect of salt concentration on colony extension on solid agar but not mycelium weight. However, the colonies on the agar may be sparser resulting in less actual biomass. This was not quantified on the agar plates and so results may not be contradictory. The data fit the calculated linear regression lines better for the solid agar growth (Fig. 2) compared to the growth in liquid broth (Fig. 1) as evidenced by the  $r^2$  values. This is also reflected in the higher variability of data in the growth in liquid broth, indicating that the solid agar growth data may be more reliable. Except for both of the NA2 isolates, there appeared to be also more of an effect of the salt on mycelium growth in liquid broth than on solid agar, if the final weight as a percentage of the non-amended controls is examined (Fig. 1). This difference was also noted by Sterne et al. (1976) and was attributed to a measure of stress on hyphal extension. On agar, differences in colony growth were noted between the two NA1 isolates but not between the two EU1 or NA2 isolates. In liquid broth, no differences were observed between the NA1 ( $P = 0.784$ ), NA2 ( $P = 0.864$ ), or EU1 ( $P = 0.221$ ) isolates within each lineage. Since only two isolates from each lineage were examined in this study, no definite conclusions can be drawn about any differences in salt tolerance between lineages and among isolates within a lineage although preliminary conclusions can be drawn that there are no obvious differences.

**Table 2**  
Percentage of *Phytophthora ramorum*-infected *Rhododendron* 'Cunningham's White' leaf disks after exposure for 1 week in water samples collected from natural sources mixed with sand infested with *P. ramorum* chlamydospores (1000 cm<sup>-3</sup>).

Source	Conductivity	pH	Infected leaf disks (%) <sup>a</sup>
Control (MES buffer)	20.5 µS	6.2	100 ± 0.0
Lake Pontchartrain	5.6 mS	6.7	100 ± 0.0
York River	30.5 mS	7.5	2.2 ± 2.2*
Chesapeake Bay	32.3 mS	7.6	4.4 ± 4.4*
Atlantic Ocean	35.9 mS	7.5	1.1 ± 1.1*

<sup>a</sup> Means are followed by the standard error (95%). Values followed by \* denote significant difference compared to the control according to Tukey's test ( $P \leq 0.05$ ).

Increasing salt levels also negatively affected germination of the different *P. ramorum* propagules tested, although even at 45 g l<sup>-1</sup> germination was not completely inhibited. The lower percentage of germinated sporangia suspended only in water may be artificially low in this study because the majority of sporangia had released zoospores when plated and only empty shells were observed. In addition, *P. ramorum* was recovered from all artificially infected leaf disks exposed to the entire range of salt concentrations tested for 4 and 7 d, regardless of the isolate used (data not shown). However, it is not known what type or proportion of propagules was in the infected tissue, although other studies consistently observe mycelium and chlamydospores (Tooley et al., 2004; Riedel et al., 2012). This demonstrates the ability of *P. ramorum* to be fairly salt tolerant, which fits in with the generalization made by Duniway (1979) that *Phytophthora* spp. have a tolerance for salinity. This is in contrast to another oomycete, *Lagenidium giganteum*, where growth was completely inhibited at 20 and 30 g l<sup>-1</sup>, depending upon the isolate (Merriam and Axtell, 1982).

Other studies (Sommers et al., 1970; Sterne et al., 1976) have investigated the effect of osmotic potential on *Phytophthora* spp. growth. The addition of NaCl reduced the osmotic potential of liquid medium, which decreased growth of the pathogen. However, as Sterne et al. (1976) demonstrated for *Phytophthora cinnamomi*, specific ions will affect growth differently even if the osmotic potential is the same. The original intent of this paper was not to investigate how osmotic potential affects *P. ramorum*, but to demonstrate what habitats may need to be surveyed or are in danger of supporting habitation of *P. ramorum*. It was believed previously that *Phytophthora* spp. were isolated from marine environments (Duniway, 1979), which can range from 33 to 37 g l<sup>-1</sup>. However, all of those species have been reclassified into the genus *Halophytophthora* (Ho and Jong, 1990). Only recently, *P. inundata* and a new species proposed as *P. gemini* sp. nov. were shown to occur under natural ecological conditions in an estuarine habitat when it was isolated from seagrass in the Netherlands (Man in 't Veld et al., 2011). Does this mean they are rare to occur in these environments? Or have we just not surveyed adequately? Surveys for *P. ramorum* primarily have been conducted in fresh water bodies, particularly outside of infected nurseries (Chastagner et al., 2010). Although *P. ramorum* was detected in a brackish creek in Washington in 2010 (G. Chastagner, unpublished), extensive sampling has not been done in estuarine (0.5–35 g l<sup>-1</sup> total salinity) or marine (33–37 g l<sup>-1</sup> salinity) bodies.

In all isolates, sporangia production and infection of leaf disks declined as the salt concentration increased (Tables 1 and 2 and Fig. 3). One avenue of *P. ramorum* dispersal is production and release of propagules from infected plant material into waterways. This is the premise for using rhododendron leaves as baits in

streams (Sutton et al., 2009). Therefore, it is important to determine if *P. ramorum* can sporulate and infect in saline environments. Results of the present study show that sporulation and infection does occur, but is negatively affected as salt concentrations increase. In all isolates, sporangia production declined as the salt concentration increased. In some isolates, sporangia production was not observed at a concentration as low as  $14 \text{ g l}^{-1}$ . This is in contrast to what was reported for *Phytophthora parasitica* (syn = *Phytophthora nicotianae*) by Swiecki and MacDonald (1991) and *Phytophthora cryptogea* (Blaker and MacDonald, 1985) in a sand environment where sporangia formed at electrical conductivity values up to 37 mS. This was above the value where sporangia formed in the present study ( $\leq 26.5 \text{ mS}$ ).

Although no sporangia were observed in isolate WSDA-1772 at salt concentrations equal and greater than  $14 \text{ g l}^{-1}$ , infection of leaf disks at corresponding higher levels of conductivity did occur (Tables 1 and 2). Since zoospores were not observed to be released from sporangia suspended in high salt concentration solutions ( $\geq 20 \text{ g l}^{-1}$ ) in our study, it is likely that the *P. ramorum* positive leaf disks exposed to these higher salt solutions were infected by mycelium growing from a germinated chlamydospore. Since *P. ramorum* was not killed in artificially infected leaf disks exposed for 7 d to a  $45 \text{ g l}^{-1}$  salt solution, it can be assumed that the *P. ramorum* negative baited disks were never infected with *P. ramorum*. Based upon all of the data in our study, it is most likely that although *P. ramorum* chlamydospores can germinate at this high salt level, subsequent growth of mycelia was inhibited such that it was unable to infect the baits during the experimental time frame. It was also noted that the pH increased slightly as the salt concentration increased, particularly when the water was amended with the sea salt at  $45 \text{ g l}^{-1}$  (Table 1). Sea water is mildly basic mainly because of bicarbonate ions (Wangersky, 1972). However, even the highest pH measured in this study should not affect zoospore activity compared with the other pH levels, which was confirmed by Kong et al. (2012b) for *P. ramorum*. In terms of propagule formation, Benson (1984) did not observe any differences in *P. cinnamomi* sporangia production above pH 4.5, however the maximum pH tested in that study was 7.0 and so the effect on propagule formation at a higher pH is not known and should be examined specifically further for *P. ramorum*.

In conclusion, the information contained in this study proves that *P. ramorum* can survive and infect in an aquatic environment across a range of salinity levels. This means that some hosts may be at risk in these environments, including those inhabiting the brackish estuarine salt marshes of the southeastern U.S. and elsewhere. An earlier study (Preuett et al., 2013) demonstrated that yaupon (*Ilex vomitoria*), Virginia creeper (*Parthenocissus quinquefolia*), sweetbay magnolia (*Magnolia virginiana*), and southern magnolia (*Magnolia grandiflora*), which all have some salt tolerance, were susceptible to *P. ramorum*. Efforts may need to be made to expand the survey area and possible host range of this devastating pathogen.

## Acknowledgments

The authors wish to thank Ms. Bridget Cantwell for her technical assistance. This research was partially funded by the United States Department of Agriculture-National Institute of Food and Agriculture 1890 Capacity Building Grant 2010-38821-21539.

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